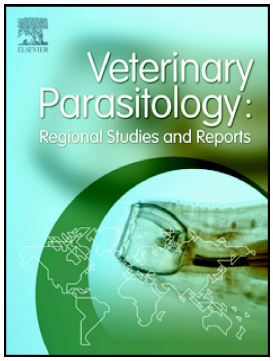
Morphological and molecular identification of Explanatum explanatum in domestic water buffalo in Pakistan



Morphological and molecular identification of Explanatum explanatum in domestic water buffalo in Pakistan

Umer Chaudhry, Bradley van Paridon, Manigandan Lejeune, Muhammad Zubair Shabbir, Muhammad Imran Rashid, Kamran Ashraf, Shoaib Ashraf, John Gilleard, Neil Sargison

PII: S2405-9390(16)30211-8
DOI: doi: 10.1016/j.vprsr.2017.02.002
Reference: VPRSR 70

To appear in: *Veterinary Parasitology: Regional Studies and Reports*

Received date: 13 October 2016
Revised date: 3 February 2017
Accepted date: 3 February 2017

Please cite this article as: Umer Chaudhry, Bradley van Paridon, Manigandan Lejeune, Muhammad Zubair Shabbir, Muhammad Imran Rashid, Kamran Ashraf, Shoaib Ashraf, John Gilleard, Neil Sargison , Morphological and molecular identification of Explanatum explanatum in domestic water buffalo in Pakistan. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Vprsr(2017), doi: 10.1016/j.vprsr.2017.02.002

Short Communication

Morphological and molecular identification of *Explanatum explanatum* in domestic water buffalo in Pakistan

Umer Chaudhry[a*], Bradley van Paridon[b], Manigandan Lejeune[c], Muhammad Zubair Shabbir[d], Muhammad Imran Rashid[e], Kamran Ashraf [e], Shoaib Ashraf [f], John Gilleard[g], Neil Sargison[a]

[a] *Roslin Institute, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Scotland, UK*

[b] *Department of Biological Sciences, Faculty of Science, University of Lethbridge, Alberta, Canada*

[c] *Canadian Wildlife Health Cooperative, Faculty of Veterinary Medicine, University of Calgary, Alberta, Canada*

[d] *Department of Parasitology, University of Veterinary and Animal sciences, Lahore, Pakistan*

[e] *Quality Operations Laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan*

[f] *Department of Animal Science, McGill University Canada*

[g] *Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary, Alberta, Canada*

*Corresponding author: Umer Chaudhry, Roslin Institute, University of Edinburgh, Easter Bush EH25 9RG, Scotland, UK. Email: uchaudhr@exseed.ed.ac.uk Tel: 00441316519244

Abstract

More than 70 species of the family Paramphistomatidae, have been identified in ruminants in different parts of the world. Most are pathogenic, causing amphistomosis. Adult flukes of this Family have a predilection for the rumen, liver or bile duct of ruminants where they may cause damage to the epithelium. Identification of adult paramphistomes to the species level based on morphology alone requires specialized knowledge, whereas, molecular genetic marker analysis is more precise and transferable. In the present study, we performed both morphological and molecular characterisation of fifteen adult flukes collected from the liver of domesticated buffalo in the Punjab province of Pakistan. The morphology of five of these flukes was examined in detail and on this basis these were identified as either *Explanatum explanatum* or *Explanatum bathycotyle*. PCR and sequencing of the ITS-2 rDNA region from these 5 flukes, plus 10 others, revealed a single haplotype in all cases. This differed by just a single nucleotide polymorphism from a previously described *E. explanatum ITS-2* rDNA sequence. Phylogenetic comparison of these *E. explanatum* ITS2-rDNA sequences with those from other *Paramphistomatidae, Fasciola and Dicrocoelium* species was performed to assesss within and between species variation and validate the use of ITS-2 rDNA as a robust species-specific marker for *E. explanatum.* This work provides a validated species-specific marker of *E. explanatum* and the first report of this parasite species from Pakistan.

Keywords: Paramphistomatidae, *E. explanatum,* ITS2, molecular marker, molecular characterization.

1. Introduction

Domesticated buffalo (*Bubalus bubalis*) are the mainstay of milk and meat production throughout Asia, where they are invariably kept in environments that include bodies of water and mud in which they wallow. There are estimated to be 23.5 million water buffalo in Pakistan, of which about 75% are in the Punjab province. The environments in which water buffalo are kept are well suited to the water and mud snails that act as intermediate hosts for a range of Fasciolidae, Paramphistomatidae, Dicrocoeliidae and Schistosomatidae trematode parasites of the liver, forestomach, intestinal tract and blood, causing disease and production loss. Immature and adult parasites of the Fasciolidae (Afshan et al., 2013; Amor et al., 2011; Shahzad W, 2012; Shoriki et al., 2014) and Paramphistomatidae families (Hanna et al., 1988; Ichikawa et al., 2013; Khan et al., 1990; Mazahery et al., 1994) are common in the liver parenchyma and bile ducts of slaughtered buffalo kept throughout subtropical regions. In addition, the Dicrocoeliidae family parasites which require a land snail first intermediate host, are occasionally reported in the livers of buffalo in subtropical regions, for example in Iran and India (Eduardo, 1985; Gorjipoor et al., 2015; Jithendran and Bhat, 1996).

Adult stages of *Explanatum* spp. infect the bile ducts, where they feed on blood across the luminal epithelium. Massive infection of intrahepatic ductules and larger bile ducts leads to the formation of multifocal granulomatous nodules throughout the luminal surfaces with grossly visible fibrosis and thickening. The feeding behavior of adult *Fasciola* and *Dicrocoelium* spp. and associated bile duct pathology is similar. In addition immature stages of *Explanatum* spp. may cause disease due to their feeding in the small intestine, while *Fasciola* spp. cause massive damage as they migrate through the hepatic parenchyma. Larval stages of *Dicrocoelium* spp. are not considered to be pathogenic while

migrating through the intestinal lumen and common bile duct (Gupta et al., 1978; Kelly, 1993).

The precise diagnosis of liver fluke infections of live animals is hindered by a lack of simple coprological tests to reliably identify the presence of immature stages. Various ELISAs have been developed to detect antibody responses, but these cannot confirm or quantify levels of current infection. While the sensitivities of coproantigen ELISAs to detect parasite secretory-excretory proteins can be helpful (Saifullah et al., 2013), but are generally inadequate for the detection of early infections in natural hosts (Gordon et al., 2012). Patent liver fluke infections can be identified by means of faecal egg counting, albeit the sensitivities of these methods applied to individual animals are poor, while their specificities are reduced by difficulties in differentiating between the eggs of different species belonging to the Fasciolidae and Paramphistomatidae families, or between eggs of *Explanatum* spp. and Paramphistomatidae species of the gastrointestinal tract. Dicrocoeliidae eggs are distinctive, but their small size hinders coprological identification (Sargison et al., 2012). Consequently production losses are commonly either inaccurately ascribed to liver fluke infections, or simply attributed to other factors such as poor weather or undernutrition, resulting in frequently irrational and ineffective disease control. Furthermore, failure to differentiate between fluke genera and species can result in inaccurate interpretation of results and consequent treatment failures (Gordon et al., 2013).

Offal are routinely inspected at abattoirs, providing an opportunity for the identification of pathology attributed to liver fluke infections. In most regions, livers identified with signs of fluke infection upon visual inspection, palpation or incision of the gastric surface are rejected. Reports of liver rejections due to fluke infections are potentially helpful in informing sustainable parasite management strategies, but underutilized in many countries, including Pakistan, in part due to concern about their

accuracy. Indeed, while Paramphistomatidae ruminal and liver flukes are seen in buffalo in Pakistan (Iqbal et al., 2014; MN Iqbal et al., 2014) and northern India (Khan et al., 2015), there are no validated reports determining their species identity. Since, different management strategies and anthelmintic treatments are required for *Fasciola*, *Explanatum* and *Dicrocoelium* spp., the value of abattoir-derived information to livestock producers depends upon both the sensitivity and specificity of reports of specific parasitic infections.

There are three validated species in the genus *Explanatum* which are considered to be present in buffalo throughout Asia: *Explanatum explanatum*, *Explanatum bathycotyle* and *Explanatum anisocotylea* (Eduardo, 1984; Ichikawa et al., 2013; Mazahery et al., 1994). These species are morphologically similar to rumen flukes of the genus *Gigantocotyle* (Rojo-Vazquez et al., 2012), of which *Explanatum* was formerly considered to be a subgenus (Eduardo, 1984). Paramphistomatidae liver flukes are seen in slaughtered buffalo in Pakistan where they are generally referred to as *Gigantocotyle explanatum* (Eduardo, 1985), albeit this species is not formally recognized (Eduardo, 1984) and is generally considered to be a synonym for *E. explanatum*. However, confirmation of the true species identity of Paramphistomatidae flukes by morphological examination can be imprecise in all except the most experienced hands, while molecular speciation methods are seldom used. *Fasciola hepatica* is generally described as occurring in temperate regions (Farjallah S, 2013; Ichikawa and Itagaki, 2010) and *Fasciola gigantica* in tropical areas (Amor et al., 2011), but both species overlap in subtropical areas along with intermediate genotypes (Agatsuma T, 2000). *F. gigantica* is the predominant species in buffalo and cattle in the Punjab and Baluchistan provinces of Pakistan (Chaudhry et al., 2015), but co-infections with *F. hepatica* and *F. gigantica* and intermediate genotypes have been described in the same region (Agatsuma T, 2000; Huang et al., 2004; Ichikawa and Itagaki, 2010; Marcilla A, 2002; Rokni MB, 2010). Dicrocoeliidae family flukes in

Pakistan are identified as *Dicrocoelium dendriticum* (pers comm Dr. Kamran Ashraf, 2014), but *Dicrocoelium hospes* and *Dicrocoelium orientalis* (Maurelli et al., 2007; Otrantoa et al., 2007) may also be present. The biology, life cycles and epidemiology of the different liver flukes parasites of Asian buffalo differ, hence there is a need for accurate identification of these parasite species.

Here we describe for the first time the molecular confirmation of *E. explanatum* infection of buffalo using rDNA ITS-2 marker, and further discuss the significance of its phylogenetic relationship to other fluke parasites. We also report the gross morphological differentiation between *Explanatum*, *Fasciola* and *Dicrocoelium* as an aid to their identification in livers of slaughtered buffalo in Pakistan.

2. Materials and Methods

2.1. Fluke collection sites

We identified two regions of Pakistan's Punjab province, where we anticipated a high prevalence Paramphistomatidae in the livers of buffalo. Infected livers were collected from three individual buffalo, slaughtered in city abattoirs, and transported to the laboratory on ice. The livers were then dissected to reveal flukes in the biliary ducts. 3, 5 and 7 adult flukes were collected from with two populations obtained from Faisalabad (E1B and E10B) and one population obtained from Okara (E8B) respectively. To describe the morphology of adult liver flukes, parasites confirmed using molecular methods, as being *F. gigantica* were derived from different regions in Pakistan (Chaudhry et al., 2015), *D. dendriticum* were derived from cattle and elk in the Cypress Hills, Alberta, Canada (Beck et al., 2014).

2.2. Morphology of liver Paramphistomatidae

Morphological examination of the Paramphistomatidae collected at the abattoirs was undertaken according to existing keys (Eduardo, 1985). Flukes were first examined under an Olympus SZ16 stereomicroscope and images were taken with DP72 digital camera. CellSens standard software (Olympus life sciences) was used to undertake morphometric measurements. Values for whole body length, maximum width, diameter of the acetabulum, width and length of testes (both anterior and posterior), length and width of ovary and the dimensions of the eggs were recorded. Body length and width were measured on un-flattened flukes, whereas other features were observed and measured with flattened, stained specimens. Morphological and morphometric data from this study were compared with those reported for previous studies (El-Rahimy et al., 2012; Ichikawa et al., 2013; Otrantoa et al., 2007).

2.3. Genomic DNA isolation, PCR amplification and sequence analysis of rDNA ITS-2

Individual flukes recovered from the abattoir derived livers were washed repeatedly in phosphate buffered saline (PBS) and preserved in 70% ethanol at -80^{o}C. For DNA extraction, about 2 mg of tissue was removed from each fluke and rinsed for 5 min in dH_2O, twice. Tissue sections were lysed in lysis buffer (50mM KCL, 10mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 0.045% Nonidet p-40, 0.45% Tween-20, 0.01% gelatin and dH_2O in 50ml volumes) and Protinease K (10mg/ml, New England BioLabs). Samples were lysed in 50µl for 98 min at 60^{o}C, followed by 15 min at 94^{o}C, and then stored at -80^{o}C until required.

A fragment of the ITS-2 rDNA region was amplified from individual adult fluke lysates using a universal forward primer complementary to the 5.8S rDNA coding sequence (5’ GGTGGATCACTCGGCTCGTG 3’) and reverse primer complimentary to the 28S rDNA coding sequence (5’ TTCCTCCGCTTAGTGATATGC 3’). These primers

were designed after the modifying previously reported primers used for Fasciolidae (Adlard et al., 1993; Chaudhry et al., 2015). Reaction mixtures of 25µl contained final concentrations of 1X Thermopol reaction buffer (New England Biolabs), 2 mM $MgSO_4$, 100 uM dNTPs, 0.1 uM forward and reverse primers and 1.25 U Taq DNA polymerase at 5000U/ml (New England Biolabs). Thermo cycling conditions were 95°C for 5 min followed by 35 cycles of 95°C for 30 seconds, 56°C for 60 seconds and 72°C for 60 seconds, with a final extension of 72°C for 5 minutes. PCR products were cleaned using Omega BioTek Micro Elute Cycle Pure Kit (D6293-02) and the same amplification primers were used to sequence both DNA strands using an Applied Biosystems 3730Xl genetic analyzer. rDNA ITS-2 sequences of both strands from each individual fluke were assembled, aligned and edited to remove primers and poor quality sequence on both ends using Geneious Pro 5.4 software (Kearse et al., 2012).

2.4. Phylogenetic analysis of the rDNA ITS-2 data set

Ribosomal DNA ITS-2 sequences obtained from of our abattoir-derived liver Paramphistomatidae, along with 24 different published sequences for Paramphistomatidae genera and species (Fig 2), and 40 different published sequences for five other representative liver fluke species (Fig. 3) were aligned and the 5.8s and 28s flanking regions trimmed using the Geneious software (Kearse et al., 2012). The flanking regions are highly conserved, so this allowed comparison of the same DNA fragment across all species. The sequences were then imported into MEGA 6 (Tamura, 2011) and used to determine the appropriate model of nucleotide substitution to build the phylogeny. The best model of substitution according to the Bayesian information criterion was the Kimura 2 model (K2+I), which was used with parameters estimated from the data. Branch supports were obtained by 1000 bootstraps of the data, with the cut-off value set at 50%

and all branches below condensed to a single node. The most probable ancestral node was determined by rooting the networks to a closely related outgroup and *H. contortus* sequence was used to root the Fluke network.

3. Results

3.1. Gross liver pathology

Severe bile duct pathology was observed in each of the three livers, consistent with previous reports of *E. explanatum* infection of buffalo (Ichikawa et al., 2013; Mazahery et al., 1994). This included clearly visible granulomatous nodules on the thickened epithelium of the bile duct where the Paramphistomatidae flukes were attached by means of their acetabulum. *Fasciola* spp. were also co-infecting within the bile ducts.

3.2. External morphology and morphometric characterization of liver Paramphistomatidae

The morphological characteristics of five Paramphistomatidae liver flukes that were prepared for examination are shown in Fig. 1a and their morphometric values are presented in Table 1. The morphological features of *Fasciola* and *Dicrocelium* are presented in Fig 1b for comparison.

3.3. Confirmation of liver Paramphistomatidae fluke species identity by phylogenetic analysis of rDNA ITS-2 sequences

281bp rDNA ITS-2 fragments were PCR amplified and sequenced from the 15 abattoir derived liver Paramphistomatidae flukes. These all showed 100% sequence identity to each other but had a single nucleotide polymorphism at position 195 when

compared with two previously published *E. explanatum* sequences derived from buffalo in Myanmar (data not shown). Twenty eight Paramphistomatidae rDNA ITS-2 sequences previously published in the NCBI Genbank public database comprise twenty five unique haplotypes, while the 15 sequences of our abattoir-derived liver Paramphistomatidae comprise a single haplotype. A maximum likelihood (ML) phylogenetic tree was made for these 26 haplotypes to examine the phylogenetic relationship between the two *E. explanatum rDNA* ITS-2 haplotypes those from the other most important Paramphistomatidae species (Fig. 2). The *E. explanatum* sequences are most closely related to *P. leydeni* and *P. epiclitum* (Fig. 2). There were 7 and 12 fixed variable nucleotide sites between the haplotype from our 15 abattoir-derived liver Paramphistomatidae and those from *P. leydeni* and *P. epiclitum* respectively (data not shown).

Thirteen rDNA ITS-2 sequences of *F. hepatica* selected from the NCBI Genbank public databases have two unique haplotypes and 14 *F. gigantica* sequences have four unique haplotypes. However 1, 2 and 8 rDNA ITS-2 sequences of *D. hospes*, *D. orientalis* and *D. dendriticum*, respectively have nine unique haplotypes. Moreover the two published rDNA ITS-2 sequences of *E. expalantum* and 15 sequences of our abattoir-derived liver Paramphistomatidae have two haplotypes. A maximum Likelihood (ML) tree was constructed from these 17 haplotypes (Fig. 3) to examine the phylogenetic relationship between the two *E. explanatum rDNA* ITS-2 haplotypes those from the other most important liver fluke species; *F. hepatica*, *F. gigantica*, *D. hospes*, *D. orientalis* and *D. dendriticum*. The *E. expalantum* haplotypes were more closely related to the *Fasciola* genus than the *Dicrocoelium* genus (Fig. 3).

4. Discussion

Accurate diagnosis of liver fluke infections both in live animals and at postmortem examination is a prerequisite to inform effective and sustainable disease control. Our description of morphological differences between the liver flukes could be adapted for use by abattoir meat inspectors to provide accurate and informative feedback to producers. This approach can be helpful in stimulating the development of targeted rational parasite control in livestock. The value of abattoir data as a basis for better understanding of the epidemiology of liver fluke parasites is further highlighted by the need to develop new control solutions due to the widespread emergence of anthelmintic resistance in Fasciolidae (Mitchell et al., 1998) Paramphistomatidae (Mas-Coma et al., 2005) and Dicrocoeliidae (Tharaldsen and Wethe, 1980) flukes.

We have described the morphology of our Paramphistomidae liver flukes using keys provided by Eduardo (1984). These allowed us to confirm the genus identity as being *Explanatum*. *E. anisocotylea* was excluded on the basis of the distance between the pharynx and acetabulum (being shortest in this species), the measurements for our flukes being consistent with those for *E. explanatum* or *E. bathycotyle*. However *E. bathycotyle* can be differentiated based on the internal morphology of terminal genitalium which is the gracile type in *E. bathycotyle*, but explantum type in *E. explanatum*. None of our flukes yielded desired sectioning appropriate to read the internal morphology, therefore, we could not confirm their internal morphology. Our flukes were slightly larger, with larger acetabulae than those previously described as *E. explanatum* (Eduardo, 1984; Ichikawa et al., 2013). However, values obtained from morphometric measurements may vary due to age of the specimen, relaxed or contracted fixation status and the angle of the preparation. These factors highlight the complementary value of molecular methods in parasite species identification.

We have combined classical morphology and molecular methods to confirm the species identity of *E. explanatum* liver flukes in slaughtered buffalo in Pakistan. The identity of *E. explanatum* has only previously been confirmed in studies of slaughtered cattle and buffalo in Myanmar (Ichikawa et al., 2013), Iran (Mazahery et al., 1994) and India (Ahmad et al., 2004). Number of studies have shown the value of rDNA ITS-2 sequence for the accurate species differentiation of Paramphistomatidae, including *E. explanatum* (Ichikawa et al., 2013); Fasciolidae including *F. hepatica* and *F. gigantica* isolates (Amor et al., 2011; Chaudhry et al., 2015; Farjallah S, 2013; Ichikawa and Itagaki, 2010; Itagaki et al., 1998) and Dicrocoeliidae including *D. dendriticum* (Beck et al., 2014; Gorjipoor et al., 2015; Maurelli et al., 2007; Otrantoa et al., 2007). Importantly, molecular analysis of the ITS-2 rDNA locus can also be used to show genetic variation in parasite species. We analyzed the ITS-2 rDNA of 15 individual *Paramphistomatidae* liver flukes taken from the livers of three buffalo to provide proof of concept intraspecific genetic variation in *E. explanatum* from Pakistan. Our full sequence analysis of 281bp of the ITS-2 rDNA locus revealed intraspecific variations at position 195. Furthermore, the Pakistani *E. explanatum* are genetically different from isolates taken from buffalo in Myanmar (Ichikawa et al., 2013). We have used previously published *D. dendriticum*, *D. orientalis*, *F. hepatica* and *F. gigantica* isolates along with the *E. explanatum* from our study to provide more information about the intraspecific sequence variations at the ITS-2 rDNA locus. The ITS-2 sequences of *D. dendriticum* showed 5 intraspecific variations (Gorjipoor et al., 2015; Otrantoa et al., 2007), while the corresponding sequences of *D. orientalis* and *D. hospes* showed 100% sequence identity (Maurelli et al., 2007; Otrantoa et al., 2007). The rDNA ITS-2 sequences of *F. hepatica* showed a single intraspecific SNP (Farjallah S, 2013), while those of *F. gigantica* showed 3 intraspecific variations (Chaudhry et al., 2015). These findings show the potential for development of molecular

population genetics tools to study the changing epidemiology of fluke parasites arising as a consequence of changing management, climatic conditions and anthelmintic resistance.

We have identified interspecific single nucleotide polymorphisms at numerous positions in the rDNA ITS-2 between sequences of *E. explanatum* and other two Paraphistomidae species, *P. leydeni* and *P. epiclitum*. The rDNA ITS-2 regions of *E. explanatum* and *P. leydeni* species differ at 7 nucleotide positions, whereas *E. explanatum* and *P. epiclitum* species differ at 12 nucleotide positions. For instance this study is the first documented report of interspecific genetic variations in the rDNA ITS-2 sequences of *E. explanatum* and other two *Paraphistomum* species. The rDNA ITS-2 regions of *D. orientalis* and *D. dendriticum* species isolated from diverse geographical locations differ at 9 nucleotide positions, with invariant fixed differences between the two species at 8 loci and a deletion in *D. orientalis* (Otrantoa et al., 2007). The corresponding regions between *D. dendriticum* and *D. hospes* differ at 31 nucleotide positions with invariant fixed differences between the two species at 25 loci and 6 deletions in *D. orientalis* (Otrantoa et al., 2007), while and *D. orientalis* and *D. hospes* show a similar pattern of interspecific variable nucleotide positions. There are 5 interspecific variable nucleotide positions in the rDNA ITS-2 region between *F. hepatica* and *F. gigantica* taken from diverse geographical regions. Four SNPs show invariant fixed differences between the two species and the final mutation is an insertion in *F. hepatica* (Chaudhry et al., 2015). Better understanding of the molecular evolutionary biology and phylogenetics of fluke parasites will help to inform novel methods for their control that are now needed. We have shown variations between the size of the rDNA ITS-2 regions of *E. explanatum, Dicrocoelium* spp. and *Fasciola* spp., which could allow practical differentiation of the genera, based on a single PCR.

Our study has described the morphology and molecular characterization of *E. explanatum* liver fluke parasites of buffalo in Pakistan. The results of our phylogenetic

analyses have implications for the diagnosis and control of liver flukes. The potential for misinterpretation and misidentification of liver flukes highlights the need for accurate genus, or even species, identification in order to understand parasite population genetics. Without such reliable identification, it is not possible to determine differential disease outcomes and epidemiology. More polymorphic genetic markers are now needed for further molecular analysis of a wide range of isolates from different host species and geographical regions, in order to better understand the genetic variability and population structure of ruminant liver flukes.

Acknowledgements

We would like to thank Natural Sciences and Engineering Research Council of Canada (NSERC) (Grant number RGPIN/371529-2209) as well as NSERC-CREATE Host Pathogen Interactions (HPI) graduate training program at the University of Calgary. We are also grateful to the Vice Chancellor (Prof. Dr. Talat Naseer Pasha) of the University of Veterinary and Animal Science Lahore Pakistan for his great support to collect samples from Abattoirs.

References

Adlard, R.D., Barker, S.C., Blair, D., Cribb, T.H., 1993. Comparison of the second internal transcribed spacer (ribosomal DNA) from populations and species of Fasciolidae (Digenea). Int. J. Parasitol. 23, 423-425.

Afshan, K., Valero, M.A., Qayyum, M., Peixoto, R.V., Magraner, A., Mas-Coma, S., 2013. Phenotypes of intermediate forms of *Fasciola hepatica* and *F. gigantica* in buffaloes from Central Punjab, Pakistan. J. Helminthol. 1-10.

Agatsuma, T. A.Y., Iwagami, M., Honzako, Y., Cahyaningsih, U., Kang, S.Y., Hong, S.J., 2000. Molecular evidence of natural hybridization between *Fasciola hepatica* and *F. gigantica*. Parasitol. Int. 49(3),231-8.

Ahmad, G., Saifullah, M.K., Nizami, W.A., 2004. Partial purification and characterization of *Gigantocotyle explanatum* somatic antigens. J. Helminthol. 78, 95-99.

Amor, N., Farjallah, S., Salem, M., Lamine, D.M., Merella, P., Said, K., Ben Slimane, B., 2011. Molecular characterization of *Fasciola gigantica* from Mauritania based on mitochondrial and nuclear ribosomal DNA sequences. Exp. Parasitol. 129, 127-136.

Beck, M.A., Goater, C.P., Colwell, D.D., Van Paridon, B.J., 2014. Fluke abundance versus host age for an invasive trematode (*Dicrocoelium dendriticum*) of sympatric elk and beef cattle in southeastern Alberta, Canada. Int. J. 3, 263-268.

Chaudhry, U.N., Van Paridon, B., Shabbir, M.Z., Shafee, M., Ashraf, K., Yaqub, T. J. G., 2015. Molecular evidence shows that the liver fluke *Fasciola gigantica* is the predominant *Fasciola* species in ruminants from Pakistan. J Helminthol. 11,1-8.

Eduardo, S.L., 1984. The taxonomy of the family paramphistomatidae Fischoeder, 1901, with special reference to the morphology of species occurring in ruminants. IV. Revision of the genus *Gigantnocotyle* Nasmark, 1937 and elevation of the subgenus *Explanatum Fukui*, 1929 to full generic status. Syst. Parasite. 6, 3–32.

Eduardo, S.L., 1985. The taxonomy of the family Paramphistomidae Fischoeder, 1901 with special reference to the morphology of species occurring in ruminants. V. Revision of the genus *Calicophoron* Stiles & Goldberger, 1910. Syst. Parasite. 7, 3–26.

El-Rahimy, H.H., Mahgoub, A.M., El-Gebaly, N.S., Mousa, W.M., Antably, A.S., 2012. Molecular, biochemical, and morphometric characterization of *Fasciola* species potentially causing zoonotic disease in Egypt. Parasitol. Res. 111, 1103-1111.

Farjallah, S, Piras ,C.M., Amor, N., Garippa, G., Merella, P., 2013. Molecular characterization of *Fasciola hepatica* from Sardinia based on sequence analysis of genomic and mitochondrial gene markers. Exp. Parasitol. 135, 471-478.

Gordon, D.K., Roberts, L.C., Lean, N., Zadoks, R.N., Sargison, N.D., Skuce, P.J., 2013. Identification of the rumen fluke, *Calicophoron daubneyi*, in GB livestock: possible implications for liver fluke diagnosis. Vet. Parasitol. 195, 65-71.

Gordon, D.K., Zadoks, R.N., Stevenson, H., Sargison, N.D., Skuce, P.J., 2012. On farm evaluation of the coproantigen ELISA and coproantigen reduction test in Scottish sheep naturally infected with *Fasciola hepatica*. Vet. Parasitol. 187, 436-444.

Gorjipoor, S., Moazeni, M., Sharifiyazdi, H., 2015. Characterization of *Dicrocoelium dendriticum* haplotypes from sheep and cattle in Iran based on the internal transcribed spacer 2 (ITS-2) and NADH dehydrogenase gene (nad1). J. Helminthol. 89, 158-164.

Gupta, P.P., Singh, P. C., Mandal, G.B.S., Grewal, G.S., 1978. A postmortem study of mortality pattern in adult buffaloes in Punjab. Indian J. Anim. Sci., 48, 323-325.

Hanna, R.E., Williamson, D.S., Mattison, R.G., Nizami, W.A., 1988. Seasonal reproduction in *Paramphistomum epiclitum* and *Gastrothylax crumenifer*, rumen paramphistomes of the Indian water buffalo and comparison with the biliary paramphistome *Gigantocotyle explanatum*. Int. J. Parasitol. 18, 513-521.

Huang, W.Y., He, B., Wang, C.R., Zhu, X.Q., 2004. Characterisation of *Fasciola* species from Mainland China by ITS-2 ribosomal DNA sequence. Vet. Parasitol. 120, 75-83.

Ichikawa, M., Itagaki, T., 2010. Discrimination of the ITS1 types of *Fasciola* spp. based on a PCR-RFLP method. Parasitol. Res. 106, 757-761.

Ichikawa, M., Kondoh, D., Bawn, S., Maw, N.N., Htun, L.L., Thein, M., Gyi, A., Sunn, K., Katakura, K., Itagaki, T., 2013. Morphological and molecular characterization of *Explanatum explanatum* from cattle and buffaloes in Myanmar. J. Vet. Med. Sci. 75, 309-314.

Iqbal, A., Muhammad, A., Anjum, K., Shahzad, M. A., S. Ali., 2014. Epidemiology of *Gigantocotyle explanatum* in naturally infected buffaloes. Veterinaria. 2, 15-18.

Iqbal, M.N., Ali, M., Aftab Ahmad, A., Khawar A.S., Muhammad A. A., Ali, S., 2014. Prevalence of *Gastrothylax crumenifer* in the gastrointestine of *Bubalus bubalis*. Veterinaria. 1, 28-31.

Itagaki, T., Tsutsumi, K.I., Ito, K., Tsutsumi, Y., 1998. Taxonomic status of the Japanese triploid forms of *Fasciola*: comparison of mitochondrial ND1 and COI sequences with *F. hepatica* and *F. gigantica*. J. Parasitol. 84, 445-448.

Jithendran, K.P., Bhat, T.K., 1996. Prevalence of dicrocoeliosis in sheep and goats in Himachal Pradesh, India. Vet. Parasitol. 61, 265-271.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 28, 1647-1649.

Kelly, 1993. The liver and biliary system. Pathology of Domestic Animals. 4th edn., Academic Press, London, UK.

Khan, Nizami, W.A., Ahmad, M, 1990. Biochemical variations in *Gigantocotyle explanatum* and *Gastrothylax crumenifer* with respect to their seasonal reproduction. Int. J. Parasitol. 20, 109-117.

Khan, Y.A., Singh, B.R., Ullah, R., Shoeb, M., Naqvi, A.H., Abidi, S.M., 2015. Anthelmintic Effect of Biocompatible Zinc Oxide Nanoparticles (ZnO NPs) on *Gigantocotyle explanatum*, a Neglected Parasite of Indian Water Buffalo. PloS one 10, e0133-086.

Marcilla, A, B.M., Mas-Coma S., 2002. A PCR-RFLP assay for the distinction between *Fasciola hepatica* and *Fasciola gigantica*. Mol. Cell. Probes. 16(5), 327-333.

Mas-Coma, S., Bargues, M.D., Valero, M.A., 2005. Fascioliasis and other plant-borne trematode zoonoses. Int. J. Parasitol. 35, 1255-1278.

Maurelli, M.P., Rinaldi, L., Capuano, F., Perugini, A.G., Veneziano, V., Cringoli, G., 2007. Characterization of the 28S and the second internal transcribed spacer of ribosomal DNA of *Dicrocoelium dendriticum* and *Dicrocoelium hospes*. Parasitol. Res. 101, 1251-1255.

Mazahery, Y., Razmyar, J., Hoghooghi-Rad, N., 1994. *Explanatum explanatum* (Creplin, 1847) Fukui, 1929, in buffaloes in the Ahwaz area, southwest Iran. Vet. Parasitol. 55, 149-153.

Mitchell, G.B., Maris, L., Bonniwell, M.A., 1998. Triclabendazole-resistant liver fluke in Scottish sheep. Vet. Record. 143, 399-400.

Otranto, D., Steffen, R., Stefania W., Cinzia, C., Antonio, P., Riccardo, P. Liaa., Olsond, P.D., 2007. Morphological and molecular differentiation between *Dicrocoelium dendriticum* (Rudolphi, 1819) and *Dicrocoelium chinensis* (Sudarikov and Ryjikov, 1951) Tang and Tang, 1978 (Platyhelminthes: Digenea). Acta Trop. 104, 91–98.

Rojo-Vazquez, F.A., Meana, A., Valcarcel, F., Martinez-Valladares, M., 2012. Update on trematode infections in sheep. Vet. Parasitol. 189, 15-38.

Rokni, M.B., Mizani, A., Mohebali, M., Sharbatkhori, M., Kia. E.B., Abdoli, H., Izadi, S., 2010. Identification and differentiation of *Fasciola hepatica* and *Fasciola gigantica* using a simple PCR-restriction enzyme method. Exp. Parasitol. 2, 209-213. .

Saifullah, M.K., Ahmad, G., Abidi, S.M., 2013. Immunodetection of coproantigens for the diagnosis of amphistomosis in naturally infected Indian Water Buffalo, *Bubalus bubalis*. Vet. Parasitol. 191, 66-72.

Sargison, N.D., Baird, G.J., Sotiraki, S., Gilleard, J.S., Busin, V., 2012. Hepatogenous photosensitisation in Scottish sheep casued by *Dicrocoelium dendriticum*. Vet. Parasitol. 189, 233-237.

Shahzad, W., Munir, R., Aslam, W., Ijaz, M., Ahmad, R., Khan MS., Sabir, A.J., 2012. Prevalence and molecular diagnosis of *Fasciola hepatica* in sheep and goats in different districts of Punjab Pakistan. Pak. Vet. J. 32, 535-538.

Shoriki, T., Ichikawa-Seki, M., Devkota, B., Rana, H.B., Devkota, S.P., Humagain, S.K., Itagaki, T., 2014. Molecular phylogenetic identification of *Fasciola* flukes in Nepal. Parasitol. Int. 63, 758-762.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5:Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance and Maximum Parsimony Methods. Mol. Biol. Evol. 28, 2731–2739.

Tharaldsen, J., Wethe, J.A., 1980. A field trial with albendazole against *Dicrocoelium lanceolatum* in sheep. Nordisk veterinaermedicin. 32, 308-312.

Figure Legends

Fig.1. (a) *Explanatum,* whole worm ventral view (scale bar = 1mm). **(b)** Fluke morphotypes (1) *Fasciola* identified based on triangular shape, prominent shoulder and converging borders (2) *Dicrocoelium* identified based on testes orientation, overall size and level of maximum body width with respect to the middle of the fluke body. (3) *Explanatum* identified based on large body, tapering anteriorly, broad and rounded posteriorly, curved ventrally.

Fig.2. Phylogenetic analysis of twenty six haplotypes are identified from 43 rDNA ITS-2 sequences of Paramphistomatidae (for more detail of sequences and accession no. see the footnote*). Twenty six haplotypes are identified from 17 sequences of *Explanatum* spp., 8 sequences of *Calicophoron* spp., 4 sequences of *Paramphistomum* spp., 3 sequences of *Orthocoelium* spp., 2 sequences of *Gastrothylax* spp., 3 sequences of *Fischoederius* spp., 2 sequences of *Homologaser* spp., and 1 sequence of *Gastrodiscoides* spp., *Carmyerius* spp. and *Watsonius* spp. All haplotypes fells in to a species specific clades, making phylogenetic relationship among them impossible to infer from their ITS-2 sequences

alone. Nonetheless, the *Explanatum* haplotypes are identified as *E. explanatum* is closest to that of the previously identified *E. explanatum* (KF564869, AB743577). The sequences were aligned by Geneious software and tree obtained by Maximum Likelihood (ML) analysis using Kimura 2 model (K2+G) model of substitution. Branches with bootstrap support values above 50% (1000 replications) and posterior probability greater than 50 respectively are represented at the base of the nodes. The phylogeny is rooted with rDNA ITS-2 sequence of parasitic nematode *H. contortus* (Genebank accession number X78803).

* *Calicophoron* spp. (accession nos. GU735641, GU735646, GU133057, AB042188, GU735656, AB056570, AY790883, GU133057), *Paramphistomum* spp. (accession nos. GU735660, HM026462, HM209067, JF834888), *Orthocoelium* spp. (accession nos. GU133058, AB042189, GU735648), *Gastrothylax* spp. (accession no. GU735659, HM159382), *Fischoederius* spp. (accession no. GU133061, GU133062 GU133059), *Gastrodiscoides* spp. (accession no. EF027097), *Velasquezotrema* spp. (accession no. HM159383), *Carmyerius* spp. (accession no. HM159381), *Watsonius* spp. (accession no. GU999987) and *Homologaser* spp. (accession no. AB042190, GU133056).), *E. explanatum* (accession nos. KF564869, AB743577).

Fig.3. Phylogenetic analysis of seventeen haplotypes are identified from 55 rDNA ITS-2 sequences of five liver flukes species (for more detail of sequences and accession no. see the footnote*). Two haplotypes (Fg-H1, Fg-H2) are identified from 13 sequences of *F. hepatica,* four haplotypes (Fg-H3, Fg-H4, Fg-H5, Fg-H6) are identified from 14 sequences of *F. gigentica,* seven haplotypes (Dd-H9, Dd-H10, Dd-H11, Dd-H12, Dd-H13, Dd-H14, Dd-H15) are identified from 8 sequences of *D. dendriticum,* one haplotype (Do-H7) is identified from 2 sequences of *D. orientalis,* one haplotype (Dh-H8) is identified from 1 sequence of *D. hospes,* one haplotype (Ee-H16) identified from 2 sequences of *E. explanatum* and one haplotype (Ee-H17) is identified from 15 sequences of *E. explanatum* selected in this study. The sequences were aligned by Geneious software and tree obtained by maximum likelihood (ML) analysis using Kimura 2 model (K2+G) model of substitution. Branches with bootstrap support values above 50% (1000 replications) and posterior probability greater than 50 respectively are represented at the base of the nodes.

The phylogeny is rooted with rDNA ITS-2 sequence of parasitic nematode *H. contortus* (Genebank accession number X78803).

* *F. hepatica* (Accession no AB207148, AJ557568, EF612479, AJ557567, AM900370, AM707030, AM709498, GQ231546, GQ231547, FJ467927, FJ593632, AB010974), *F. gigantica* (Accession no AJ853848, AJ557569, EF612482, AB010977, AB207151, EF612484, AM900371, EU260063, AB010976, KM259915, KM259916, KM259917, KF667375, AB553710), *D. dendriticum* (JQ966972, HM026461, HM358027, DQ379986, AB367789, AB369980, AB369981 and unpublished sequence), *D. orientalis* (EF547132, unpublished sequence), *D. hospes* (EF102026) *E. explanatum* (K564869, AB743577).

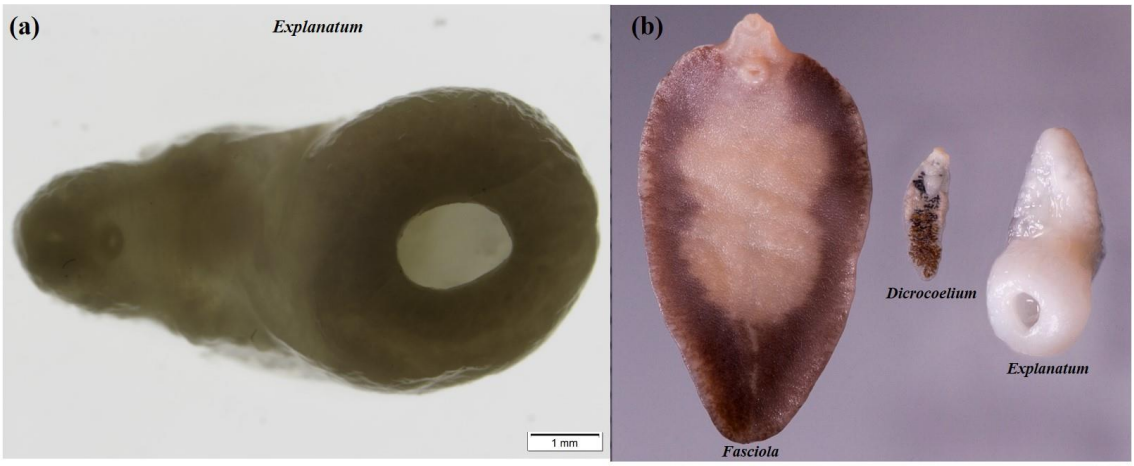


Fig. 1

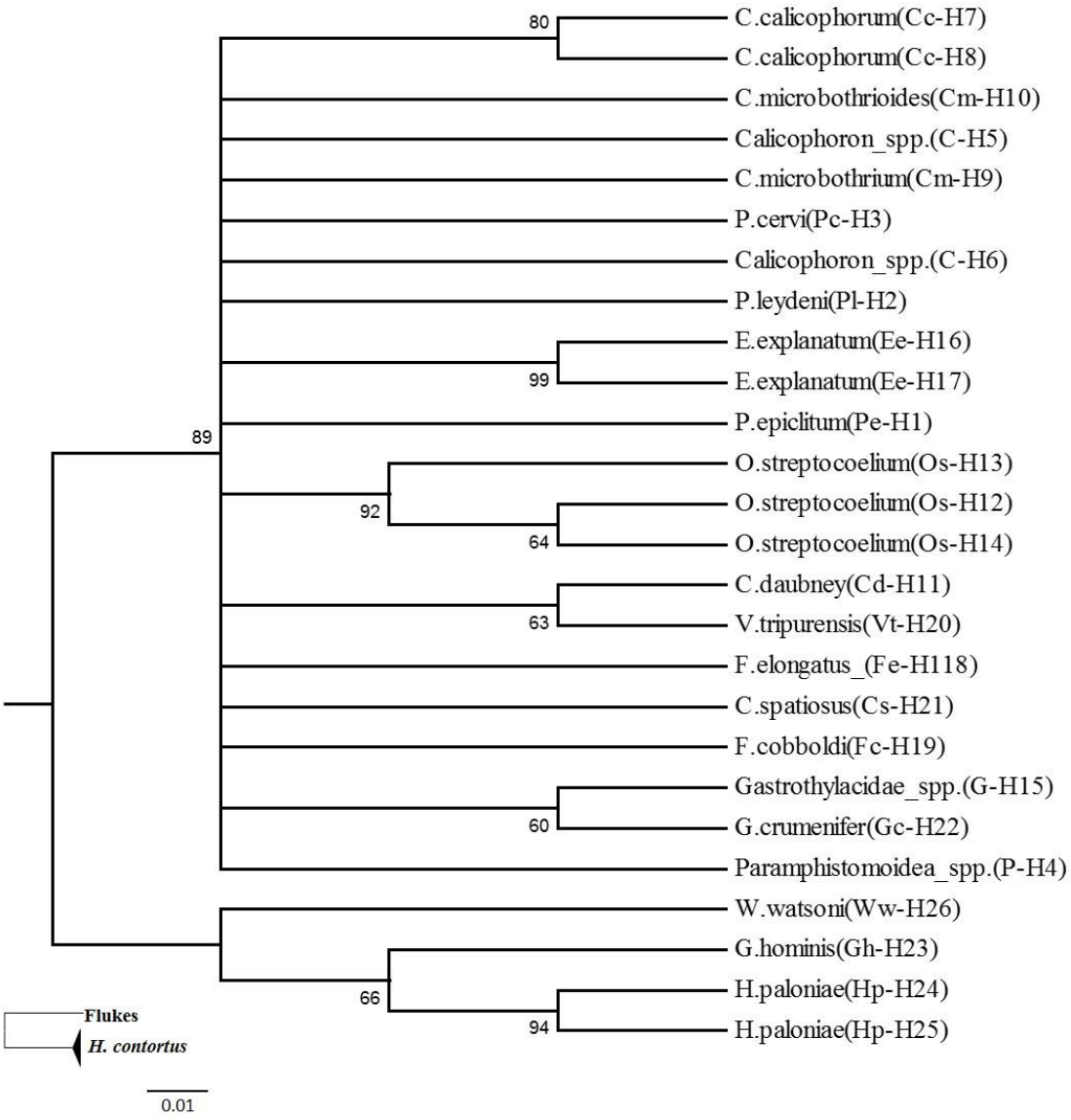


Fig. 2

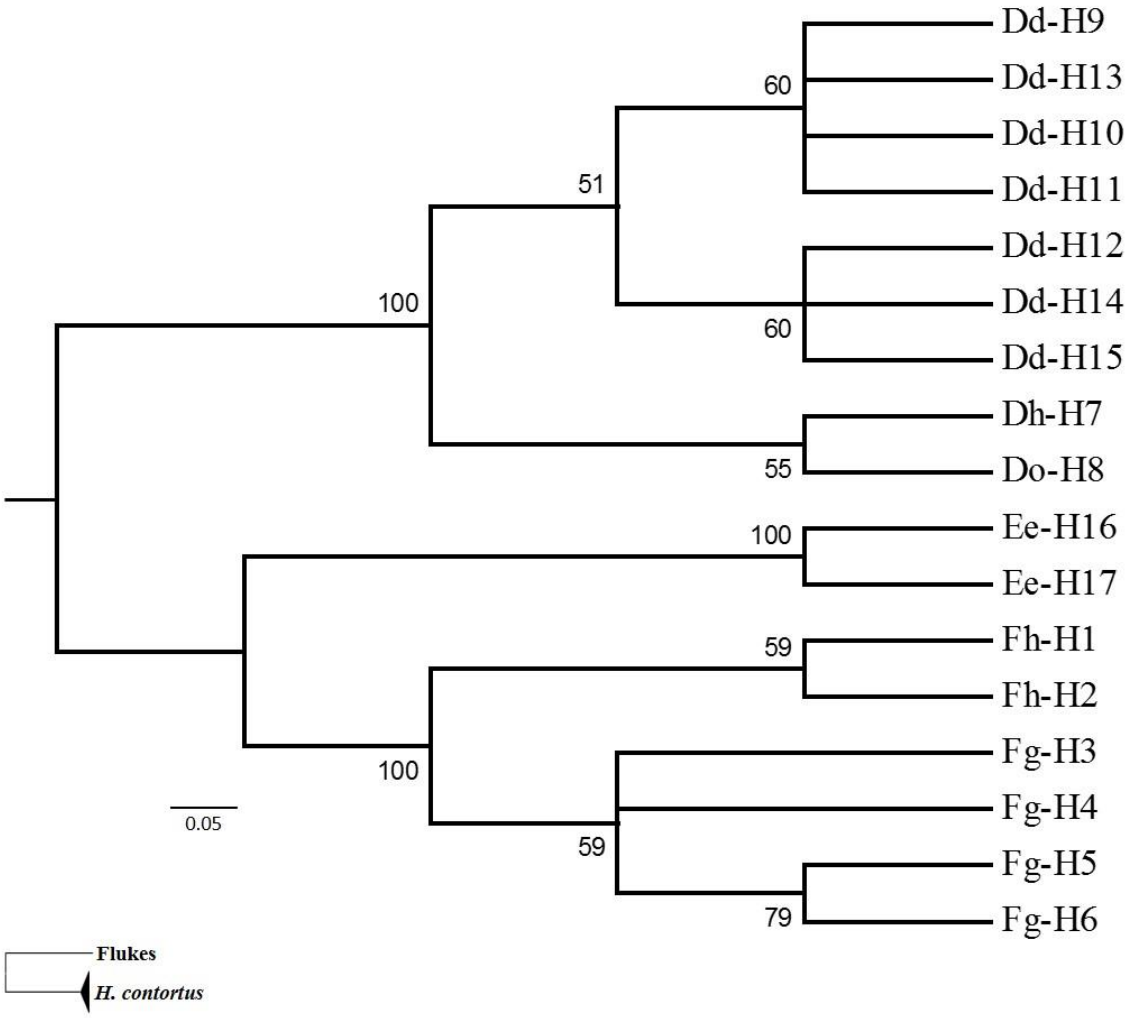


Fig. 3

Table 1: Morphometric value of *Explanatum* measured in our study.

	Explanatum
Body length (mm) n=5	10.00-11.9
Body width (mm) n=5	4.3-5.8
Acetabulum (mm) n=5	3.8-5.4
Anterior testes (mm) n=4	
Width	2.85-3.78
Length	1.29-2.31
Ovary (mm)n=2	
Width	0.96-1.37
Length	1.85-2.25
Egg n=5	
Length (µm)	128.51-143.0
width(µm)	84.55-96.2

Highlights

- Describe for the first time the molecular confirmation of *E. explanatum* infection of domesticated water buffalo using rDNA ITS-2 marker, and discuss the significance of its phylogenetic relationship to other fluke parasites.
- This is the first report of the *Explanatum explanatum* in Pakistan in the peer-review literature.
- We reported the gross morphological differentiation between *Explanatum*, *Fasciola* and *Dicrocoelium* as an aid to their identification in livers of slaughtered buffalo in Pakistan.